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Interleukin-8 expression by mammary gland endothelial and epithelial cells following experimental mastitis infection with *E. coli*

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Abstract

Epithelial and endothelial cells play a pivotal role in initiating and controlling the movement of leukocytes into tissues during inflammation through the production of cytokines and chemokines such as interleukin-8 (IL-8). In situ hybridization with an IL-8 riboprobe was used to determine IL-8 mRNA expression by mammary gland epithelial and endothelial cells in cows with experimental *Escherichia coli* mastitis. Epithelial cells of the gland, especially surrounding the alveoli, had increased IL-8 mRNA levels at all time points at which tissue samples were collected (8, 12, and 24 h) after *E. coli* challenge. Levels of IL-8 expression in the epithelial cells decreased at 24 h post-infection. IL-8 expression by mammary gland endothelial cells was low, but did increase slightly at 24 h post-infection. Both epithelial and endothelial cells of the mammary gland can contribute to the production of IL-8 that is typically seen in coliform mastitis.

Keywords: Coliform mastitis; Interleukin-8; Mammary gland; Epithelial cell; Endothelial cell

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Résumé

Les cellules épithéliales et endothéliales jouent un rôle majeur dans l'initiation et le contrôle des mouvements leucocytaires dans les tissus inflammés, ceci grâce a des cytokines et des chemokines telles que l'interleukine 8 (Il 8). En utilisant une technique d'hybridation in situ avec ARN complémentaire, l'expression d'ARNm de l'Il 8 fut mesurée dans les cellules épithéliales et endothéliales des glandes mammaires de vaches pendant une mamite expérimentale à coliformes. Les cellules épithéliales mammaires, en particuliers autour des alvéoles, présentèrent un niveau élevé d'ARNm d'Il 8 à tout moment de l'experience (8, 12 et 24 heures post-infection). L'expression d'Il 8 diminua à 24 heures post-infection. Le niveau d'ARNm d'Il 8 était bas dans les cellules endothéliales mammaires, mais augmenta à 24 heures post-infection. Les cellules épithéliales et endothéliales des glandes mammaires peuvent contribuer à la production d'Il 8 qui est typique et observé dans la mastites coliforms.

Mots clés: Mastite coliforme; L'interleukine 8; Glande mammaire; Cellule épithéliale; Endothéliale cellule

1. Introduction

Interleukin-8 (IL-8) is an important chemokine involved in the attraction of neutrophils to sites of inflammation. Several animal models have demonstrated increased IL-8 levels at sites of inflammation. Examples of this include lung and broncheo-alveolar lavage (BAL) samples from calves experimentally infected with *Mannheimia haemolytica*; joint fluid from dogs infected with *Borrelia burgdorferi* (Lyme's disease), and macrophages isolated from the joints of goats infected with caprine arthritis encephalitis virus [1–5]. Inhibition of IL-8 impairs neutrophil migration in response to bacterial products (e.g. LPS), chemical irritants (e.g. hydrochloric acid infused into the lungs of rabbits and mice, respectively), and in cardiac ischemia-reperfusion injury in rabbits [6–8]. Interleukin-8 has direct effects on bovine neutrophils as well. Recombinant bovine IL-8 is a chemoattractant for bovine neutrophils in both microchemotaxis chambers and following intradermal injection in vivo [9]. In addition, IL-8 directly stimulates bovine neutrophil phagocytosis, priming, enzyme degranulation, and oxygen radical production [10–12].

Interleukin-8 appears to be one of several inflammatory mediators involved in inflammation and leukocyte migration into the mammary gland during bovine mastitis. The migration process occurs relatively early in mastitis, with migration of leukocytes out of post-capillary venules at 2 h and into the teat sinus at 4–6 h after infection [13]. During this period, levels of IL-8 in milk are increased [14–16]. In addition, somatic cell numbers in the milk mirror the increased levels of IL-8 and other inflammatory mediators (e.g. complement component C5a, TNF-α, IL-1β, IL-12, IFN-γ, and IL-10) in the milk following infection with the Gram negative bacteria *Klebsiella pneumoniae* and *Escherichia coli* [17–21]. Neutralization of IL-8 in milk samples from cows infected with *Staphylococus aureus* reduced neutrophil migration in an in vitro chemotaxis assay [22]. A similar result was shown by Li et al. who found the chemotactic acitivty of IL-8 in milk samples from cows that had been infused in the mammary gland with endotoxin were neutralized with either an anti-IL-8 antibody or an IL-8 antagonist [23]. Recombinant human IL-8 is chemotactic for bovine neutrophils in vitro, although direct infusion of

rhIL-8 into the mammary gland did not lead to increased somatic cells in the milk [24,25]. In contrast, a recent study by Takahashi et al. demonstrated significantly increased somatic cell numbers in bovine mammary glands naturally infected with *S. aureus* that were concurrently infused with recombinant bovine IL-8 [26]. It is possible IL-8 infusion into the teat sinus alone does not stimulate migration of neutrophils into the teat cistern, but likely works in concert with other factors (e.g. other cytokines, bacterial products) to create effective chemotactic gradients within the tissue.

The source of IL-8 in the mammary gland during coliform mastitis is not well defined. Several cell types in the gland have the potential to produce IL-8, including resident macrophages, epithelial cells lining the alveoli and ducts, endothelial cells, and leukocytes that migrate into the gland during the infection [27–29]. Messenger RNA for other cytokines, such as TNF-α, IL-6, and IL-12, were increased in somatic cells isolated from cows with experimental *Staphylococcus* mastitis at 24 h after infection [29]. A likely source of IL-8 early in the infection process (<24 h) would be the epithelial and endothelial cells lining the alveoli, ducts, and surrounding blood vessels. The objective of this study was to determine the relative contribution of mammary gland epithelial and endothelial cells to IL-8 production early in *E. coli* mastitis using in situ hybridization to analyze mammary gland tissue samples obtained from cows experimentally infected with *E. coli*.

2. Material and methods

2.1. Animals and tissue preparation

All animals used were housed and provided humane care under the USDA-ARS National Animal Disease Center Animal Care and Use Committee approved protocol no. 1898. Two of the Holstein cows used in this study originated from the NADC mastitis research herd and were not vaccinated with *E. coli* J5 vaccine. The additional four Holstein cows were purchased from a local university research herd and had entered the NADC Mastitis research herd 4 months prior to this study. The vaccination history of these animals prior to entering the NADC herd is not known. However, because the effects of the J5 vaccine are short-lived, the vaccination status of these animals should not have affected the scores. The cows were in various stages of lacation, which ranged from 35 to 200 days in milk at the time of challenge. The ages of the cows ranged from 2 to 5 years. All cows were free from known mastitis infections at the time of the challenge on the basis of milk culture samples taken prior to and at the time of challenge.

The six cows were injected in the teat canal of the right front quarters of the udders after the morning milking with 20–40 colony forming units of *E. coli* MacDonald strain 487 resuspended in 2 ml pyrogen free saline. The 487 strain was originally isolated from a cow with naturally occurring acute mastitis in the NADC mastitis research herd. The left front quarter of the udders of the same cows were infused in the teat canal with 2 ml pyrogen free saline as a negative control. Milk somatic cell numbers were monitored prior to and following challenge. In addition, milk culture samples were taken prior to and every 2 h after infection. The animals were euthanized at 8 h (two cows), 12 h (two cows), and 24 h (two cows) by overdose of phenobarbital IV. Mammary glands from the cows were

removed and necropsied. Sections of mammary tissue were collected (lactiferous sinus and mammary parenchyma), fixed in 10% zinc buffered formalin, and stored in 75% ethanol until being embedded in paraffin. The blocks of tissue were sectioned and mounted on slides for H&E staining and in situ hybridization.

2.2. Riboprobe construction

Probe design, synthesis, and labeling was performed using techniques as described by Brown and from the book: Nonradioactive In Situ Hybridization Manual [30,31]. The bovine IL-8 DNA sequence was located using a BLAST (Basic Local Alignment Search Tool) search through PubMed (National Institutes of Health, Bethesda, MD, USA). The software program Primer Express (Applied Biosystems, Foster City, CA, USA) was then used to develop a forward and reverse primer set to be used in producing an amplicon for IL-8 riboprobe development. The primer sequences were synthesized by the University's Biotechnology Center (UW Madison, Madison, WI, USA). PCR was done to amplify the DNA sequences flanked by the two primer sets using the PCR Core System 1 kit (Promega, Madison, WI, USA), a thermalcycler (Peltier Thermal Cycler-200, MJ Research, Sierra Point, CA, USA), and an available cDNA library for the template. The IL-8 sequence amplified was as follows:

cacacctttctacccccaaatttatcaaagaattgagagttattgagagtgggccacactgtgaaaattcagaaatcatgt
aagttatttcaaaagtgattattttactttagtcagcctagaattgagacgtggaagaatccagcaaagttctaggtactaggagt
acatagtgagaaaaatagaaaggaaaaattctttgtctccatggcatttaatatgggactctaatagctaaaaattagtttgga
cttccattttatgcctgtactcaaggaaccatgacttgaatggcaaggtggt

The resulting IL-8 amplicon was ligated into the pGEM®-T Easy Vector (Promega) following the protocol described in technical manual no. 042: pGEM®-T and pGEM®T Easy Vector Systems [32]. This vector was used in the transfection reaction with JM109 bacteria (L2000A, Promega). Transfected bacteria were grown on selective media, after which several positive colonies were selected and transferred to and grown in LB broth containing ampicillin. After an overnight incubation at 37 °C, the plasmid was isolated from the bacteria using the QIAprep Minikit (Qiagen, Valencia, CA, USA). A sample of the isolated plasmid was sequenced by the University's Biotechnology Center (UW-Madison) to confirm insertion and orientation of the IL-8 sequence. Additional transfected bacteria containing the sequenced plasmid were grown in LB broth with ampicillin, and the plasmids were recovered using the Qiagen plasmid Midikit (Qiagen). The recovered plasmid was used for transcription of the riboprobe. Enzyme restriction sites were chosen using the software program DNAid 1.8 (Frédéric Dardel, Laboratoire de Biochimie, Ecole Polytechnique, Palaiseau Cedex, France) to cut the plasmid on either side of the IL-8 sequence. The preliminary riboprobe was constructed using a modified procedure from Promega Corporation technical manual no. 16: Riboprobe® in vitro Transcription System [33,34]. Briefly, the DNA template was digested by incubating the template with either the restriction enzyme PstI (Promega) or AatII (Promega). Phenol:chloroform:isoamyl alcohol (25:14:1) (Sigma, St Louis, MO, USA) extraction followed by ethanol precipitation was used to purify the digested plasmid. The plasmid was blunt end digested using DNA polymerase I large (Klenow) fragment (Promega). Labeling consisted of using either a SP6 or T7 RNA polymerase (Promega) with DIG labeled

nucleotides (Boehringer Ingelheim, Ridgefield, CT, USA). After the labeling reaction, the labeled RNA probe was recovered using ethanol precipitation, dried under vacuum, and resuspended in nuclease-free H_2O. A dot blot was done to check for digoxigenin-labeled riboprobe incorporation into the plasmid.

2.3. In situ hybridization

The tissue samples described above were used for in situ hybridization using a modified protocol [33,35]. Briefly, the slides were heated to 70 °C for 10 min, deparaffinized in Citrisolv (Fisher, Pittsburgh, PA, USA) (3 min × 3), and then air dried. Tissues were encircled with a hydrophobic barrier pen and rehydrated with PBS + 5 mM $MgCl_2$ for 10 min at 23 °C. Slides were rinsed in 0.2 M Tris pH 7.5 + 0.1 M glycine for 10 min at 23 °C and then incubated with pepsin (98.3 ml H_2O, 1.7 ml 12 N HCl, 0.1 g Pepsin) (Sigma) in 10 mM Tris pH 7.5 + 2 mM $CaCl_2$ for 30 min at 37 °C. The enzyme activity was stopped with 0.2 M Tris pH 7.5 + 0.1 M glycine for 2 min × 2. Slides were incubated with the prehybridization solution (12.5 ml 20× SSC, 0.5 ml 10% *N*-laurosarcosine, 0.1 ml 10% SDS, 24.0 ml formamide (Sigma), 2.5 g Northern blocking reagent, final volume 50 ml with RNase-free H_2O) for 1 h at 42 °C. The prehybridization solution was drained off and siliconized coverslips were spotted with hybridization fluid (prehybridization solution + sense/antisense probe, ≈25 ng probe/slide, 100 μl total). Slides were inverted onto the coverslip and the edges of the coverslip were sealed with nail hardener. Slides were incubated overnight in a humid chamber at 37 °C. On the second day of the ISH procedure, the coverslips were removed and slides were washed with 2× NaCl/sodium citrate (SCC) with 1% sodium dodecyl sulfate (SDS) for 30 min at 45 °C, 1× SSC with 0.1% SDS for 30 min at 45 °C, 1× SSC three times for 10 min each at 23°, and 0.1× SSC for 15 min at 23 °C. Slides were then washed in Buffer 1 (150 nM NaCl + 100 mM Tris pH 7.5) for 5 min, followed by incubation for 2 h at 37 °C with anti-dig antibody diluted 1:300 in 2% normal sheep serum in Buffer 1 (100 μl/slide). This was followed by three washes for 15 min at 23 °C in Buffer 1 and 5 min at 23 °C in Buffer 3. For visualization, a substrate (2 ml Buffer 3, 9 μl nitro blue diformazan (NBT), 7 μl 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP)) and 1 drop 5 mM Levamisole (Sigma) was added to the slides, which were incubated in the dark. Slides were checked microscopically at intervals to monitor development.

After the in situ hybridization procedure was completed, the slides were counterstained with light green stain (Sigma), and mounted with coverslips. The slides were examined with a light microscope (Labophot, Nikon, Melville, NY, USA) at 100× magnification. Areas of the slides containing a high density of blood vessels were observed and the stained epithelial and endothelial cells were scored on a gradient scale of 0, no staining of any cells; 1, faint positive staining of 1–50% of cells; 2, intense positive staining of 1–50% of cells; 3, faint positive staining of >50% of cells; 4, intense positive staining of >50% of cells; 5, 100% positive staining of all cells. Each slide was evaluated by three independent observers using the same gradient criteria. The results from these counts were averaged and the combined data represented graphically to visualize expression trends. Photographs of representative slides were taken to document the results (Fig. 1).

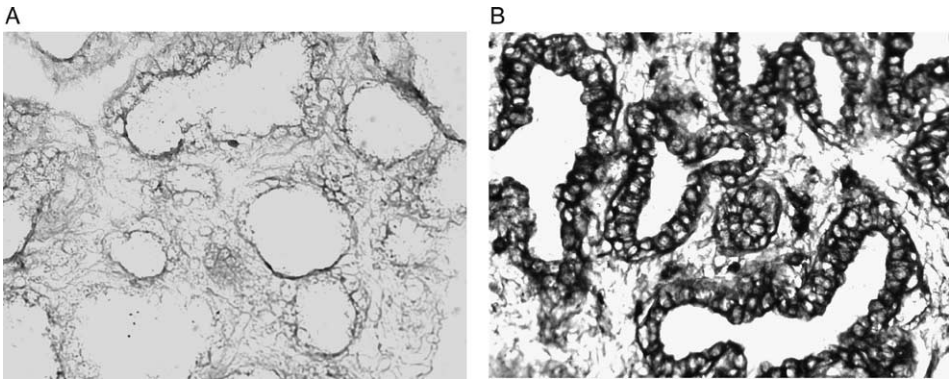

Fig. 1. Staining of mammary gland parenchyma sections, obtained from cows 12 h after *E. coli* inoculation with sense and antisense IL-8 riboprobes. Tissue sections were mounted on glass slides, depariffinized, lightly digested with pepsin, and then exposed overnight to either the DIG labeled sense or antisense IL-8 riboprobes. After several washes with SSC, the tissues were incubated with a HRP conjugated anti-DIG antibody followed with exposure to the chromagen BCIP and NBT. Samples shown are representative of (A) negative staining with the sense IL-8 riboprobe and (B) positive staining with the IL-8 antisense riboprobe.

2.4. Statistical analysis

Comparison of staining intensity scores among the three observers was evaluated by McNemar's Symmetry χ^2-test and Cohen's Kappa statistic using the Crosstabs function in the Tables Module of SYSTAT 10.2 (SYSTAT Software, Inc., Richmond, CA, USA). Based on the observer agreement findings, the approach adopted was to abandon the five-level scale and to dichotomize the staining intensity scores as above or below the median (median = 1) of all 275 scores. Scores equal to one or less were coded as 0 and scores greater than one were coded as 1. This binary score was used as the dependent variable in fitting a logistic regression model using the LOG IT Module of SYSTAT 10.2. The main effects measured in the original study design that included time (8, 12 or 24 h), probe (IL-8 sense and IL-8 antisense), challenge status of the gland (challenge or control), tissue location (lactiferous sinus or parenchyma), and cell type (alveolar epithelium, sinus epithelium, or endothelium). In the revised analysis, slide rater identity (1, 2 or 3) was included as a main effect in the logistic model. By including rater identity as a main effect, the odds ratio estimates of the other main effects were adjusted for differences among the three observers. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Clinical response

Somatic cell counts and milk cultures were performed to ascertain the degree of mastitis in the *E. coli* infected quarters. All *E. coli* infected quarters exhibited increasing

somatic cell counts 3 h after infection as compared to the control quarters (data not shown). In addition, the earliest positive milk culture was at 8 h after infection, and only from the quarters infected (data not shown). At the time of necropsy, erythema was present in the mammary parenchyma of all challenged glands, and was not present in the contralateral quarter at all time points. Milk secretions in the milk sinuses were also abnormal based on the presence of flakes and clots. At 12 and 24 h there was evidence of swelling and hardness of the challenged gland.

3.2. IL-8 mRNA expression in the samples

Two tissue sections (lactiferous sinus and parenchyma tissue) were probed by in situ hybridization per cow with both the antisense and sense IL-8 riboprobes and then graded by the observers. The results of the grades were averaged and the combined data are presented. There was a significant increase in IL-8 mRNA signal in the sections probed with the antisense riboprobe compared to the sense riboprobe (data not shown). The data illustrated in Fig. 2 suggests increased IL-8 mRNA signal in infected quarters. However, the small number of animals used and the wide grading scale (0–5) precluded a rigerous statistical analysis of the data. However, when this scale was modified and the data were scored as above (value of 1) or below (value of 0) the mean value of all 275 samples evaluated, a significant effect of inoculation was observed ($P < 0.05$). IL-8 mRNA levels were higher in epithelial cells in the sinus tissue sections from the infected quarters as compared to saline-inoculated controls at all time points. IL-8 levels were also higher in epithelial cells from parenchymal tissue sections in the infected quarter compared to the saline-inoculated controls at the 8 and 12 h time points. At 24 h, alveolar epithelial cells still exhibited higher levels of IL-8 mRNA as compared to the cells from the saline-inoculated control. However, the epithelial cells lining the ducts in these sections had lower levels of IL-8 mRNA than the saline inoculated controls at 24 h. Levels of IL-8 transcript in epithelial cells lining the alveoli and ducts were decreased at 12 and 24 h as compared to the tissue from animals infected for 8 h. Levels of IL-8 mRNA signal in endothelial cells from the infected quarters were less prominent at all time points compared to the epithelial cells, al*though this difference was not statistically significant.* Levels of IL-8 mRNA were increased slightly in the endothelial cells at the 24 h time point as compared to 8 and 12 h in both the sinus and paranchymal tissue sections. There were very few resident inflammatory cells or somatic cells present on the slides; therefore, these cells were not graded for IL-8 mRNA expression.

4. Discussion

Despite a considerable amount of research examining the role of various inflammatory mediators on leukocyte migration into the bovine mammary gland, there are still questions regarding which cells are involved in producing these mediators. Epithelial cells that are involved in the secretion of milk line the various ducts of the mammary gland, and are one of the most numerous cells in the gland. In addition, this location makes them one of the first cells in the gland to come into contact with bacteria or bacterial products during the

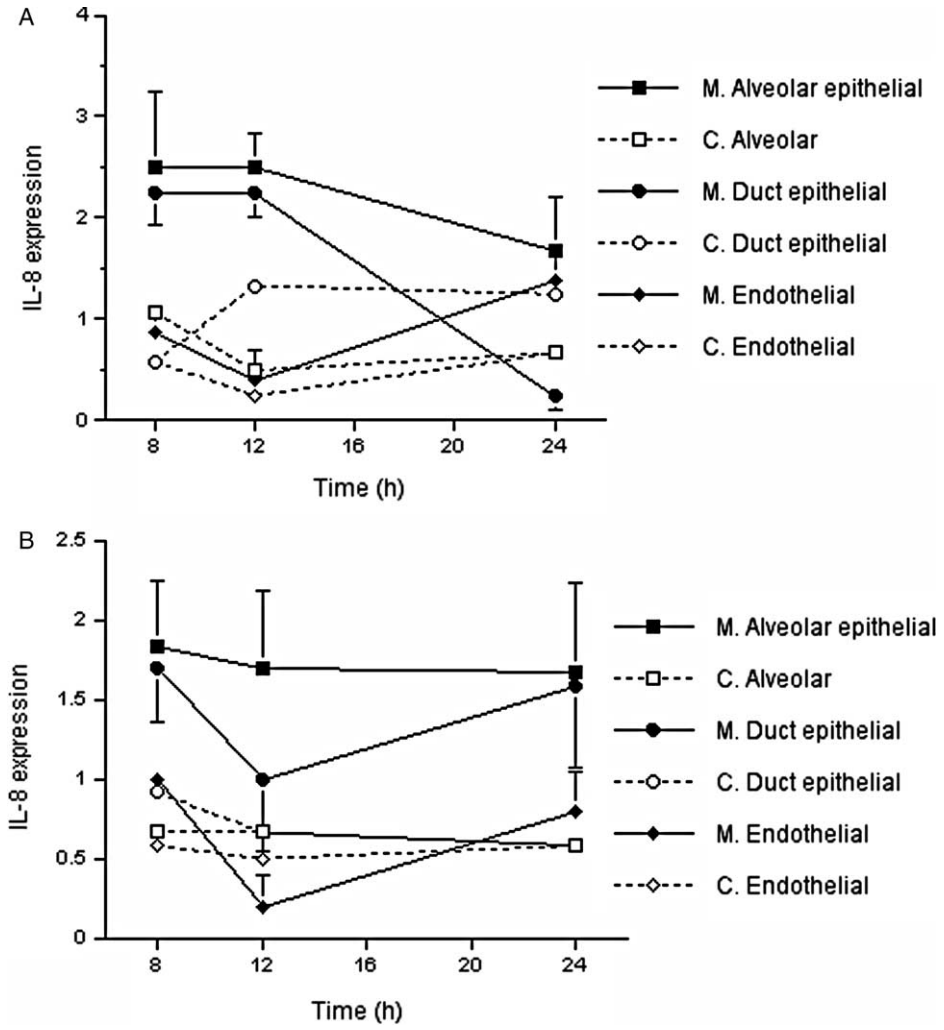

Fig. 2. Mean score for epithelial and endothelial cells in tissue sections from infected quarters stained with the IL-8 antisense riboprobe. Cells were identified and the level of staining scored in a blinded fashion by three separate observers. Values indicated on the graph are the mean ± SEM scores of the three readings at the indicated time points from tissue sections from either the (A) parenchyma or (B) lactiferous sinus of the cows. Closed symbols with solid lines are values from the *E. coli* infected quarters and open symbols with dashed lines are from the saline-inoculated control quarters. Squares represent values from epithelial cells surrounding the alveoli, circles represent values from epithelial cells lining ducts, and diamonds represent values from endothelial cells.

onset of mastitis. Endothelial cells, lining the extensive vascular network of the mammary gland, may also contribute to the production of inflammatory mediators during mastitis. One likely important inflammatory mediator produced in the gland during mastitis is IL-8. Experimental mastitis infections with *E. coli* have demonstrated measurable IL-8 levels in the milk as early as 8 h and a peak between 16 and 18 h after infection [36–38].

Epithelial cells lining the alveoli had the highest levels of IL-8 mRNA in the infected quarters, when compared to the other cells visualized in tissue sections. We assume that Increased IL-8 mRNA transcripts in these cells likely results in greater IL-8 production. Cultured epithelial cells exposed to endotoxin secrete measurable levels of IL-8 between 6 and 12 h after LPS exposure, with IL-8 levels increasing up to 48 h after exposure [27]. In the mammary gland, some of the IL-8 likely exits the gland and enters the lymphatic vessels. In one study, IL-8 could be detected in the lymph as early as 2 h post-infusion with endotoxin [16]. In the present study, epithelial cells followed a similar pattern, with the highest IL-8 mRNA levels being detected at the 8 and 12 h after inoculation, and then decreasing by 24 h. Epithelial cells located in other organs of the cow exhibit a similar pattern when exposed to Gram negative bacteria. For example, epithelial cells from the lung exhibited increased IL-8 production within 2 h after exposure to *M. haemolytica* [2,3].

Endothelial cells have also the ability to upregulate cytokine production after stimulation. Some of the inflammatory mediators released by activated endothelial cells include IL-1, IL-6, IL-8, and GM-CSF [39]. Endothelial cells likely contribute to leukocyte activation via IL-8 release during inflammation. Topham et al. demonstrated that IL-1 stimulated endothelial cells could stimulate neutrophil degranulation within 4 h, and much of this activity was due to IL-8 production by the endothelial cells [40]. Other stimuli, such as TNF-α, ischemia/reperfusion injury, and bacterial products (i.e. LPS) can stimulate endothelial cells [41,42]. Exposure of human lung microvascular and umbilical vein endothelial cells to endotoxin caused increased production of IL-8 as compared to control endothelial cells [42]. It appears that most of the IL-8 remains associated with the endothelial cell membrane, where it can interact directly with leukocytes in the circulation [41,43]. In the present study, endothelial cells in the infected quarters had IL-8 mRNA scores that were lower than for epithelial cells in the same quarters, although this difference was not statistically significant. In addition, there was a small increase in IL-8 mRNA scores in the endothelial cells at 24 h as compared to the 8 and 12 h time points.

In the present study mammary gland quarters infected with *E. coli* had significantly increased levels of IL-8 mRNA as compared to the saline-inoculated quarters. The increases in IL-8 mRNA levels were greatest in the alveolar epithelial cells at the 8 and 12 h time points, which would correlate with the peak levels of IL-8 found in the milk of cows with experimentally induced mastitis [36–38]. Therefore, mammary gland epithelial cells, and to a lesser extent, endothelial cells, may be important early sources of IL-8 during coliform mastitis.

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